Fish & Shellfish Immunology 26 (2009) 908–912

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

Cloning and characterization of the trout perforin

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ARTICLE INFO

Article history:
Received 23 December 2008
Received in revised form
19 March 2009
Accepted 30 March 2009
Available online 12 April 2009

Keywords:
Pore-forming toxin
MACPF superfamily
Perforin
Evolution
Rainbow trout
Expression
Phylogenetic analysis

ABSTRACT

The pore-forming protein, perforin is one of the effectors of cell-mediated killing. A perforin cDNA clone was isolated from rainbow trout (*Oncorhynchus mykiss*) after screening of a spleen cDNA library. The full-length cDNA is 2070 bp in size, encoding for a polypeptide of 589 amino acids. The predicted amino acid sequence of the trout perforin is 64, 58 and 40% identical to those of Japanese flounder, zebrafish and human perforins, respectively. Although its membrane attack complex/perforin (MACPF) domain is conserved, trout perforin shows low homology to human and trout terminal complement components (C6, C7, C8 and C9), ranging from 19 to 26% identity. Expression analysis reveals that the trout perforin gene is expressed in the blood, brain, heart, kidney, intestine and spleen. Phylogenetic analysis of proteins which belong to the MACPF superfamily clusters the trout perforin in the same group with other known perforins.

1. Introduction

Pore-forming toxins (PFTs) are proteins that possess the ability to switch from a water-soluble form to a membrane-inserted pore form [1]. Although PFTs have been considered traditionally as virulence factors contributing to bacterial invasion and infection, these molecules also play key roles in eukaryotes (e.g. viral infection, tumor surveillance) [2]. Pore-forming toxins are broadly classified into two groups depending upon whether membrane spanning is achieved using α-helices (α-PFTs, e.g. colicin) or β-strands (β-PFTs, e.g. pefringolysin O) [3].

One of the largest families of the β-PFTs is the MACPF superfamily; so named because of a domain common to proteins of the mammalian membrane attack complex (MAC) and perforin (PF) [4]. These molecules (C6, C7, C8α, C8β, C9 and perforin) perform crucial roles in the defense against bacterial and viral infection as well as in tumor surveillance [2]. Biochemical studies have revealed that the MACPF domain is required for membrane insertion and pore formation of C8α and C9, respectively [5].

In 1984, perforin was characterized as a lytic PFT produced by natural killer cells (NK) and cytotoxic T lymphocytes (CTL) [6]. Perforin is stored in cytoplasmic secretory granules and is released on contact to kill virus-infected or transformed cells [2]. Perforin monomers insert themselves into the plasma membrane of target cells, polymerize into pore-forming aggregates in the presence of calcium, and form transmembrane channels. Pores formed by perforin range from 5 to 20 nm in internal diameter and cause osmotic lysis of the target cells [7]. Perforin itself is able to lyse and kill cells by necrosis; however it also permits delivery of pro-apoptotic proteases (granzymes) into the target cell [8]. Analysis of perforin-deficient mice has revealed that perforin is an essential part of the main mechanism of cell lysis by cytotoxic lymphocytes [9]. The X-ray structure of the Plu-MACPF (a MACPF domain containing protein from *Photorhabdus luminescens*) [10], the MACPF domain of C8α [11] and the MACPF domain of C8α in complex with C8γ [12] revealed that the MACPF domain is homologous to the N-terminal portion of cholesterol-dependent cytolysins (CDCs). In particular, conservation of a complex core fold, including the membrane spanning clusters, termed transmembrane helices (TMH1–2), strongly suggests that MACPF proteins and CDCs form pores using an analogous mechanism.

The development of powerful informatic tools such as PSI-BLAST now permits the identification of a large number (>500) of MACPF proteins. Predictably, many of these proteins appear to be involved in immune defense of attack while only a small part of them, as C8α, C9, perforins and sea anemone toxins have been demonstrated to have lytic activity [3].

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doi:10.1016/j.fsi.2009.03.021

The characterization and the full-length nucleotide sequences of the perforin gene have been reported from three mammalian species: human, rat and mouse [13–15]. Sequences from other mammalian species, as well as from chicken and frog perforins have been deposited in the GenBank database. The perforin gene has been isolated from the Japanese flounder (*Paralichthys olivaceus*) and analyzed its hemolytic activity [16]. Moreover, sequences from zebrafish and pufferfish perforins have been deposited in GenBank database.

To gain further insight into the evolution of the MACPF gene family, we report here the primary sequence, the tissue expression profile and the phylogenetic analysis of the perforin gene from rainbow trout (*Oncorhynchus mykiss*).

2. Materials and methods

2.1. Isolation of trout perforin cDNA

Degenerated oligonucleotides were designed based on conserved homologous regions of Japanese flounder and human perforin sequences: sense 20mer, TPERFF1: 5′-GCNGGNGA(G/A)GGNTT(C/T)GA(C/T)AT-3′ based on AGEGFDI amino acid residues and anti-sense 20mer, TPERFR1: 5′-CCNCCNA(A/G) (C/T)TTNAC(T/C) TTNGT-3′ based on TKVKLGG amino acid residues corresponding to 43–49 and 323–329 amino acid residues of the Japanese flounder perforin (Accession number: BAC76420), respectively (the symbol N represents the mixture of nucleotides: A, T, C and G). These primers were subsequently applied in an RT-PCR reaction, using the one-step RT-PCR kit (Qiagen). Trout spleen total RNA was used as template. One cycle was conducted at 50 °C for 30 min. Thirty cycles were conducted, using a PCR thermocycler under the following program: 95 °C for 1 min, 47 °C for 1 min and 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. The PCR product of the expected size (850 bp) was subjected to electrophoresis in a 1% agarose gel, gel-purified (QIAquick, Qiagen), ligated into the T/A cloning vector pGEM-T easy (Promega) at 4 °C overnight and transformed into *E. coli* DH5a subcloning competent cells (Invitrogen). Positive clones were selected, recombinant plasmid DNAs were extracted (mini-prep kit, Roche) and sequenced.

A spleen cDNA library was prepared from total RNA extracted from a single trout spleen, as previously described [17]. 2×10^5 λgt10 recombinant phages of trout spleen cDNA library were screened under high stringency conditions (65 °C) using an α-^{32}P labelled cDNA probe corresponding to the 850 bp amplification product of trout perforin, described above. The probe was labelled using the random primed DNA labelling kit (Boehringer Mannheim). Positive phage plaques were picked up and cultured. The recombinant phage DNA corresponding to the longest clone of trout perforin, 2.0 kb in size, was isolated and the insert cDNA was subcloned into pGEM-T easy vector and sequenced. All sequences were determined at least twice for both strands.

2.2. Databases search/multiple sequence alignment/phylogenetic tree analysis

NCBI-BLAST (www.ncbi.nlm.nih.gov/blast) was used for detection of known orthologs and for comparison of the obtained cDNAs and deduced polypeptides. Multiple alignments were generated using the Clustal W program and the obtained alignments were used to construct phylogenetic trees using the neighbour joining (NJ) algorithm within MEGA version 4 [18]. Branch points were validated by 1000 bootstrap replications and all the other conditions were set as 'default'. The prediction of the 'domain' architecture of trout perforin protein was executed by SMART software (http://smart.embl-heidelberg.de/).

2.3. RT-PCR analysis

Total RNA was extracted from different trout tissues of a single organism, using the Nucleospin RNA II kit (Macherey-Nagel). Two and a half micrograms of total RNA from blood, brain, heart, intestine, kidney, liver and spleen were reverse transcribed (total volume 20 μl) using the oligodT primed cDNA 1st strand synthesis kit (TAKARA). The obtained cDNA (1 μl) was applied into a PCR reaction using the specific oligonucleotides TPERFF2: 5′-AGAAGGGATCAAAAAGTGGG-3′ and TPERFR2: 5′-GTCACAGACTG CACCGTTCC-3′ corresponding to nucleotides 601–620 and 882–901, respectively, of the obtained trout perforin cDNA sequence. The above primers are found in different exons of trout perforin gene, based on the exon–intron organization of the Japanese flounder perforin gene. The program was as follows: 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, followed by a final extension step at 72 °C for 10 min. Trout β-actin mRNA (positive control) was amplified using the following primers: β-actinF: 5′-CACCTTCTACAATGAGCTGC-3′ and β-actinR: 5′-AGGCAGCTCGTAGCTCTTCT-3′, as described above. DNA amplification products were analyzed by electrophoresis on a 2% agarose gel.

3. Results and discussion

Previous studies have indicated the existence of cytotoxic activity in fish, as shown by the presence of CTL-like and NK-like cells [19,20]. More recently it has been shown that Japanese flounder perforin is functionally similar to those of mammals in being part of the antiviral defence system [16]. Over the last few years, we have also isolated and characterized the terminal complement components (C6, C7, C8, and C9) genes in rainbow trout [21–26], which along with perforin belong to the same MACPF superfamily. In order to gain further insight into the evolution of the MACPF family, we have isolated and characterized the perforin gene from rainbow trout.

A full length cDNA clone of 2070 nt, named trout perforin 1, was obtained after screening of a trout spleen λgt10 cDNA library. The cDNA sequence contains a 5′-UTR of 197 nt, followed by an open reading frame of 1770 nt encoding for a polypeptide chain of 589 amino acid residues and a 3′-UTR of 103 nt containing two putative polyadenylation signals and a polyA tail. The Kozak sequence – (A/GNNAUG) – recognized by ribosomes as the translational start site, is found within the 5′-UTR sequence of trout perforin mRNA (data not shown) [27]. The sequence has been deposited in the EMBL database under accession number AM295251 (cDNA trout perforin 1).

The predicted open reading frame of 589 amino acids starts with a hydrophobic sequence, characteristic of a putative signal peptide sequence (Met-1 to Ser-23) (Fig. 1). Trout perforin exhibits the same 'domain' architecture as the mammalian orthologs, containing two conserved domains, the MACPF domain corresponding to 164–366 amino acid residues and the calcium-binding domain (C2 domain) corresponding to 413–519 amino acid residues. MACPF domain is homologous to the N-terminal portion of CDCs, and structural data reveal that MACPF comprises the putative perforin transmembrane sequence. On the other hand the C2 domain seems to be responsible for the initial interaction with the membrane [2]. Alignment of trout perforin with the mammalian counterparts shows that 20 Cys residues are well conserved (Fig. 1). Two potential *N*-linked glycosylation sites were observed at amino acid positions 373–375 and 576–578 of